Exploring the efficacy of AMACR, ERG, and AR immunostains in prostatic adenocarcinoma and their association with novel grade groups

Mohamed O. Andarawi,[1] Hassan Otifi,[1] Hesham Hassan,[1,2] Adil A. Yousif,[3] Saadalnour A. Mustafa,[4] Shawgi A. Elsiddig,[5] Asaad Ma Babker,[6] Elryah I. Ali,[7] Omer Osman Elhag[8]

[1]Pathology Department, College of Medicine, King Khalid University, Abha, Saudi Arabia
[2]Department of Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt
[3]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, Abha, Saudi Arabia
[4]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Najran University, Saudi Arabia
[5]Clinical Laboratory Sciences Department, College of Applied Medical Sciences, Jouf University, Sakaka, Saudi Arabia
[6]Department of Medical Laboratory Sciences, College of Health Sciences, Gulf Medical University, Ajman, United Arab Emirates
[7]Department of Medical Laboratory Technology, College of Applied Medical Sciences, Northern Border University, Arar, Saudi Arabia
[8]Department of Histopathology and Cytology, Faculty of Medical Laboratory Sciences, Omdurman Islamic University, Sudan

ABSTRACT

The study examines the utility of AMACR, ERG, and AR immunostains in diagnosing prostatic adenocarcinoma (PCa) and assessing prognosis in comparison to the Gleason score and new WHO grading groups. Seventeen PCa biopsies and five benign prostatic hyperplasia (BPH) biopsies were analyzed. Immunoreactivity, scored from 1 to 3 based on percentage of positive cells and intensity of expression, was assessed, revealing 76.47% positivity for AMACR, 35.29% for ERG, and 94.12% for AR in PCa cases, with variable scores and intensity among markers and grade groups. AMACR sensitivity and ERG specificity were noted. Higher-grade PCa exhibited increased positivity for both markers, indicating prognostic significance. In BPH cases, AMACR showed positivity in 2 cases, ERG in 1, and AR in all cases, albeit with lower expression. Differential expression was observed among immunomarkers and grade groups of malignancy. AMACR and ERG stains serve as sensitive and specific markers for PCa diagnosis and prognosis. Their increasing positivity with higher-grade groups underscores prognostic value. These findings highlight the importance of immunostains in refining PCa diagnosis and prognostication.

Key words: prostatic adenocarcinoma; Gleason score; AMACR; ERG; AR; PSA.

Correspondence: Asaad Ma Babker, Department of Medical Laboratory Sciences, College of Health Sciences, Gulf Medical University, Ajman, United Arab Emirates. E-mail: asaad@gmu.ac.ae

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Introduction

Prostate cancer is the most common malignancy among men globally.[1] It has become a serious health concern in developed and developing countries.[2] As stated by previously published research,[3] the incidence of prostate cancer in Saudi Arabia was 7.7 per 100,000 men in 2008, while the mortality was 5.1 per 100,000 men, according to the International Agency for Cancer Research.[4] According to statistical data, prostate cancer is ranked sixth among Saudi men. However, the percentage of prostate cancer in Saudi Arabia is considered less than in other Gulf countries and Western countries.[5] Almost all men with prostate cancer are asymptomatic until the tumor has advanced, and typical symptoms include major crossovers with benign prostate conditions. The diagnosis of prostate cancer is challenging in many aspects. The popular screening test with serum PSA has its limitations as it is elevated even in many common benign conditions of the prostate with prostatomegaly. The interpretation of needle core biopsies in hematoxylin and eosin (H&E)-stained sections also has a diagnostic dilemma with several benign mimickers, giving rise to the need for basal cell immune markers, which have limitations. Present diagnostic tests are limited in terms of substantial false positive and false negative rates.[6] Molecular diagnostics is a rapidly emerging field of surgical pathology that is increasingly changing diagnostic and prognostic significance for various tumors. Several molecular markers may be helpful for prostate cancer risk assessment, diagnosis, and prognosis. These include markers identified through cytomorphology and immunohistochemistry,[7] those associated with cancer spread mechanisms,[8] and proteins involved in significant signalling pathways such as EGFR and HER2/NEU.[9]

The current study aims to evaluate the specificity and sensitivity of the latest immunohistochemical stains for the diagnosis of prostate carcinoma (PCa) and its utility as prognostic parameters. Though the H&E-stained histopathologic examination of prostatic biopsy is the gold standard for the initial diagnosis and categorizing the carcinoma using the latest 2016 WHO grading system, there are still limitations related to differentiating malignancy from common benign, atypical lesions like atypical adenomatous hyperplasia, atrophy, high-grade PIN. The interobserver and intraobserver variability in grading is also another challenge affecting the prognosis.[10] Negative basal cell markers with overexpression of Alpha-methyl acyl-CoA racemase (AMACR) were useful biomarkers in addressing the diagnostic dilemma.[11] As the sensitivity of AMACR stain varies in various literature from 62-100%,[12] and as also some precursor lesions and benign mimickers tend to be positive,[12,13] we aim to study the correlation of AMACR with other recent biomarkers using ERG and AR for their role in accurate diagnosis and prognosis. The score and intensity of these stains are correlated to Gleason's scoring and the new WHO grading system AMACR. Even though AMACR is typically overexpressed in prostate cancer, it is not restricted to it but is also present in up to 92% of colorectal adenocarcinomas, as well as breast, lung, ovarian, renal cell carcinomas (especially the papillary variant), as well as bladder urothelial and adenocarcinomas.[14,15] Thus, this marker is not helpful in the differential diagnosis of prostate cancer from other malignancies.

ERG (Ets-related gene product): although ERG expression lacks sensitivity in primary PCa (with 50% negatives), it appears to be quite specific for prostatic origin. More specifically, the genomic translocation has not been found in any other carcinoma, whereas the protein level is slightly less indicative since ERG expression is seen in vascular tumors, thymomas, and gynecological neoplasms.[16,17] It is also possible that the sensitivity in prostate cancer metastases exceeds that of primary tumors since TMPRSS2-ERG rearrangement might be more prevalent in metastases.[18] AR (androgen receptor): PSA and PSMA are both targets of androgen signaling, and the AR itself is also regulated in prostate cancer.[19] Again, the diagnostic use of AR staining is greatly hampered by the expression of AR in other human tissues and tumors, and it can therefore no longer be recommended.[20]

Materials and Methods

The study specimens involved a total of 25 formalin-fixed paraffin-embedded blocks of prostatic biopsy from patients. We included male patients aged 45 years and older who presented with clinical symptoms or signs suggestive of prostatic adenocarcinoma (PCa), supported by elevated serum PSA levels and imaging findings. Exclusion criteria included patients with prior treatment for prostate cancer, those with non-acinar prostate cancer variants, and cases with insufficient biopsy material. Among 25 cases, 20 were PCa, and 5 were benign prostatic hyperplasia. The samples consist of transrectal ultrasound (TRUS) guided biopsies of the prostate in 18 patients and transurethral resection of the prostate (TURP) in 7 patients with an enlarged prostate. The patient's demographic profile and clinical and laboratory data are collected from the medical records unit. The Hand E-stained slides are re-examined by another pathologist for repeat Gleason scoring and to classify as per new grade groups. Three carcinoma cases were excluded from the study as two of them showed significant crush artifacts and one of them was inadequate tissue for immunostains. Further study included 17 samples of histologically definitive PCa and 5 samples of benign prostatic hyperplasia (BPH) as benign control for immunohistochemical stains. Among malignancy specimens, 15 were needle core biopsies, and two were transurethral resection of the prostate. The rare variants of adenocarcinoma, ductal carcinoma, and patients on cancer treatment were not included.

Immunohistochemistry

Five-µm thin sections were stained with AMACR, ERG, or AR immunostains and independently interpreted by two pathologists. To block endogenous peroxidase activity and prevent interference with immunostaining, samples were treated with a 3% H_2O_2 solution at room temperature for 20 min. Following this treatment, sections were subjected to heat-induced antigen retrieval in sodium citrate buffer (10 mM, pH 6.0) for 40 min, performed at 95°C.This dual approach ensures that the immunostaining results accurately reflect the intended markers without interference from endogenous enzymatic activity. One positively charged unstained slide 5 µm thickness section was prepared for immunostains. Primary antibodies were added to the sections using ready-to-use monoclonal rabbit antibody against AMACR (Clone 13H4), ERG (Clone EP111), and AR (Clone AR441) (Dako, Glostrup, Denmark), and incubated for 45 min at room temperature. The secondary anti-rabbit and anti-mouse horseradish peroxidase (HRP) conjugated antibodies (anti-rabbit HRP: Abcam, Cambridge, UK; catalog number ab6789; anti-mouse HRP: Thermo Fisher Scientific, Waltham, MA, USA; catalog number A12345) were diluted at 1:200 in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA). These diluted secondary antibodies were applied to the samples and incubated for 1 h at room temperature in a humidified chamber to prevent evaporation. After incubation, the samples were thoroughly washed three times for 5 min each with TBS to remove any unbound secondary antibodies. Subsequently, the labelled streptavidin-biotin complex (Dako-cytomation, catalog number K0681) was added according to the manufacturer's instructions and incubated for 30 min at room temperature. The enzymatic reaction was developed using 3,3′-Diaminobenzidine as the chromogen, with incubation for 5 min at room temperature in

the dark, resulting in strong and permanent brown staining characteristic of the streptavidin-biotin immunoperoxidase technique (Dako-cytomation, catalog number K3468).[21,22] Positive and negative controls were performed simultaneously. The negative control was established by omitting the primary antibodies during the immunostaining process to assess non-specific binding and ensure the validity of the staining results. Counterstaining was made using Mayer's hematoxylin and bluing with Scott's tap water. Finally, the slides were mounted using Dibutylphthalate Polystyrene Xylene (DPX) and independently interpreted by two pathologists. The immunoreactivity of the antibodies was assessed using a semi-quantitative scoring method based on both the proportion of positively stained tumor cells and the staining intensity. Each protein's expression was evaluated by calculating a total immunoreactive score, which is derived from the product of the proportion and intensity scores. The staining proportion score reflects the estimated fraction of positively stained tumor cells, categorized as follows: 0 (none), 1 (1%-10%), 2 (11%-50%), and 3 (>51%). The intensity score quantifies the expression intensity, with values of 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong) (Table 1). The Gleason scores and grade groups of PCa, along with the immunostaining interpretations, were tabulated and analyzed.

Results

In this section, we present the immunohistochemical findings from our analysis of PCa, focusing on the expression levels and patterns of AMACR, ERG, and AR in the studied samples. Data analysis was made using SPSS ver.20. The P-value of< 0.05 was considered statistically significant. The 17 cases of unequivocal prostate acinar adenocarcinoma (PCa) and 5 cases of adenomyomatous hyperplasia were observed in Hand E sections. The carcinoma patients were aged between 57 and 82 years, with 12 patients between 70 and 79 (70.58%). Their total PSA level varies between 2.1 ng/mL to 153 ng/ml with 3 cases less than 10 ng/mL, 2 cases with PSA between 11 to 20 ng/mL, and 70.58% with PSA >20 ng/ml. All GG5 patients showed a PSA value of above 20 ng/mL except one (PSA 8.1 ng/mL). The BPH patients were aged between 46 to 83 years, with their PSA level varying from 3.86 to 14.9 ng/mL. Among PCa patients, the majority (11 cases-64.7%) belong to grade group (GG) 5 with 8 cases of Gleason score (GS) – 9 and 3 cases of GS-10. There were 4 cases of GG1, one each in GG3 and GG4.examples of staining in benign hyperplasia and PCa cases are shown in Figures 1, 2, and 3. AMACR was immunoreactive in 13 cases (76.47%) of carcinoma with a 3+ score in 10 cases (76.9%) and 2+ in 3 cases. The score was 3+ in GG5 (8/10) compared to the high 3+ score in GG1-GG4 (2/3) (Figure 2). The intensity of stain was also higher in GG5 with marked intensity in 4 out of 10 positive cases, compared to lower grades with no marked intensity. Two negative stains were seen in GG1, and one negative each in GG3 and GG5. The stain was also positive in two hyperplasia cases but with a lower score of 1+ and 2+ and mild intensity in both. ERG was positive in only 6 cases (35.29%) of carcinoma among GG4 (1 case) and GG5 (5 cases). Comparing positivity among GS of 6-8, where ERG was positive in 16.66 % (1/6) to GS of 9-10, where ERG was positive in 45.45 % (5/11) (Figure 3). Among ERG positivity in GG5, the marked intensity was seen in 40 % (2/5). In 3 cases (50%) positivity revealed a 3+ score with vigorous intensity. One hyperplasia case also showed positivity but with a 1+ score and mild intensity. AR stains were positive in almost all carcinoma cases except one case of GS9 (94.12%). Higher staining scores of 3+ and strong intensity were noted in lower GS (6-8) with 5 cases (83.33%), compared to 3 cases (30%) of 3+ among GG5. The stain was positive in all BPH benign cases but with mild to moderate intensity (Figure 1). Overall AMACR appears more sensitive marker (76.47%) and ERG appears more specific (80%) for PCa. AR is found to be non-specific for primary PCa in prostate biopsies (Table 2). The odds ratios of prostate adenocarcinoma were five higher when they were positive for AMACR and 2.18 higher when they were positive for ERG compared with being negative for these markers, respectively. The odd ratio of PCa positive for AR compared with negative was not calculated because almost all cancerous and benign tissues were positive for AR (Table 3). The Gleason grading value of prostate cancer was positively and insignificantly correlated to the staining of the AMACR (p=0.082), and it was negatively correlated to the staining of AR at a significance level of (p=0.189) (Table 4).

Discussion

Prostate cancer is the second leading cause of cancer-related death in the United States of America. Though there has been a progressive increase in adenocarcinoma of the prostate for the last few years in some countries, in the USA, there is a slight reduction in incidence due to a decline in routine PSA screening, but an increase in late-stage disease.[19] We observed a significant increase in the occurrence of PCa presenting with advanced clinical disease, which correlates with elevated serum PSA levels and higher histologic grade groups. Among the cases evaluated, only 6 (35.29%) were classified as malignancies within grade groups (GG1-GG4). Additionally, recent findings by Schafer *et al*.[23] indicated that there are notable trends in prostate cancer incidence and mortality that may align with our observations. Jain *et al*.[21] reported in their study of 26 prostate cancer cases that most patients presented in their eighth decade of life, with 30.76% of all cases belonging to Gleason scores 8-10. Our surge of patients in high grade might be due to a lack of routine PSA testing, making patients presenting with clinical disease. Most of our patients also manifested in the

Table 1. The scoring method is based on the proportion of positively stained and the staining intensity of tumor cells.

Grade group	GS	No.	AMACR score	AMACR intensity	ERG score	ERG intensity	AR score	AR intensity
GG1	6(3+3)	4	1-3+,1-2+,2-0	1-2+,1-1+, 2-0	-	-	3-3+,1-1+	3-3+,1-2+
GG2	7(3+4)	0	0	0	0	0	0	0
GG3	7(4+3)	1	0	0	0	0	3+	3+
GG4	8(3+5)	1	3+	1+	3+	3+	3+	2+
GG5	9(4+5,5+4)	8(5+3)	6-3+, 1-2+,-0	3-2+,2-1+,2-3+,1-0	1-3+,2-1+	1-3+,2-1+	2- 3+,4-2+,1-1+	4-2+,3-1+,1-0
GG5	10(5+5)	3	2-3+,1-2+,	1-2+,2-3+	1-3+,1-1+	1-3+,1-2+	1-3+,2-2+	2-2+,1-1+
Total	-	17	13	13	6	6	16	16
BPH	-	5	1-2+,1-1+	2-1+	1-1+	1-1+	1-3+,3-2+,1-1+	4-2+,1-1+

eighth decade.

There appears to be a need for molecular markers in PCa diagnosis and prognosis for a couple of reasons like a diagnostic dilemma with mimickers of carcinoma, interobserver variability in Gleason grading, and upgrading of carcinoma prostatectomy and tumor heterogeneity.[22,24] Jiang *et al*.,[25] in 2001, found AMACR as a very useful immunohistochemical stain for the diagnosis of PCa with 100% sensitivity regardless of Gleason grading, but they also noted focal positivity in 12% benign lesions. Later studies revealed AMACR reactivity of PCa in 62-100%, particularly lower positivity rate in variants of PCa like foam cells, pseudohyperplastic, and atrophic types. They also noticed positivity in benign mimickers like partial atrophy, nephrogenic adenoma, and atypical adenomatous hyperplasia.[26] Kumaresan *et al*.[10] observed AMACR positivity in 23 of 25 cases (92%) of PCa and 2 cases of weak positivity (12.5%) among 16 benign lesions. Our study showed an AMACR sensitivity of 76.47% and a specificity of only 60%. There was an increasing positivity trend in high-grade carcinoma of GG5 (10/11 cases) compared to lower-grade groups (3/6 cases). The expression score and intensity of staining are also higher in high-grade groups. The specificity was low as 2 out of 5 cases of benign prostatic hyperplasia were noted to be immunoreactive. However, in benign cases, both the expression score and intensity of stain are low. We incorporated ERG immunostain to compare its role to that of AMACR. Earlier studies showed ERG positivity in 50-70% of prostate cancer and predicted its positivity proportional to increased risk of progression of malignancy.[27,28] Others observed

Table 2. Sensitivity and specificity of markers staining.

	AMACR	ERG	AR
Sensitivity	76.47%	35.29%	94.12%
Specificity	60%	80%	0%

Table 3. The odds ratio of the positive and negative adenocarcinoma markers.

Variables	ERG	AMACR	AR
Numerator			
Positive			
Adenocarcinoma	6	13	16
BPH	1	2	5
Ratio	6	6.5	3.2
Denominator			
Negative			
Adenocarcinoma	11	4	1
BPH	4	3	0
Ratio	2.75	1.3	0
Odds ratio	2.18	5	NA

Table 4. Pearson correlations between the studied markers and Gleason score.

Markers	Pearson	*p*
AMACR	0.434	0.082
ERG	0.084	0.749
AR	-0.335	0.189

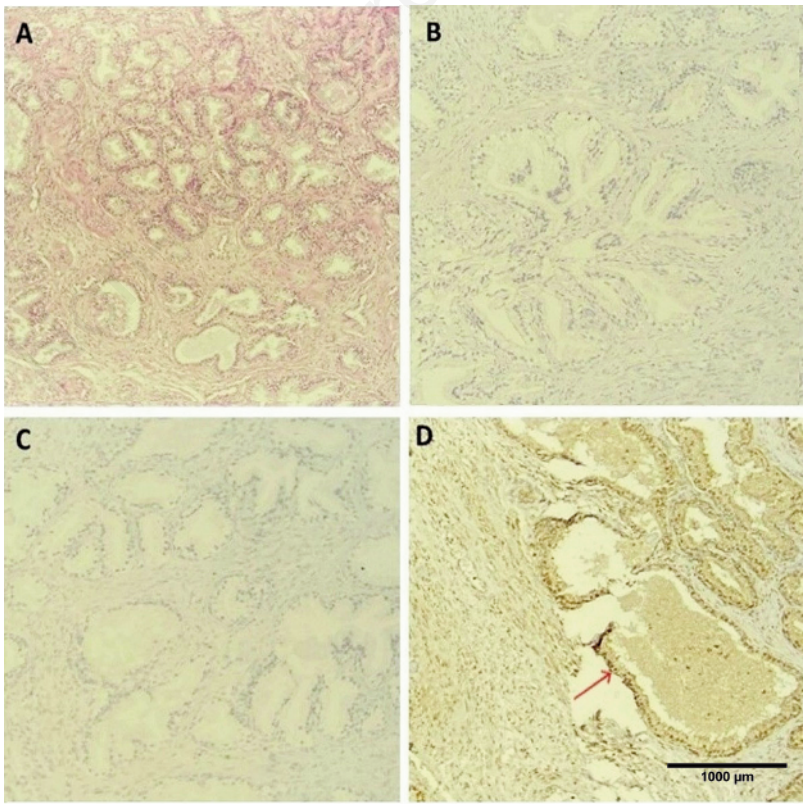

Figure 1. Histological sections of benign prostatic hyperplasia and prostatic adenocarcinoma. **A**) Benign prostatic hyperplasia stained with H&E. **B)** Negative AMACR immunoreactivity in benign tissue. **C**) Negative ERG immunoreactivity in benign tissue. **D**) Positive AR immunoreactivity in adenocarcinoma, with the arrow indicating areas of expression.

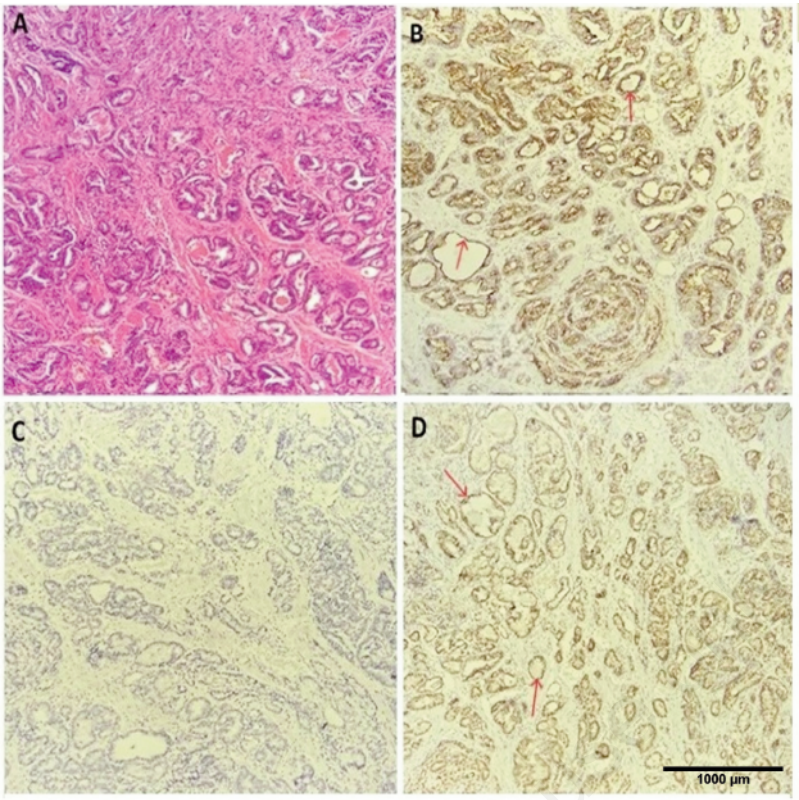

Figure 2. Prostatic adenocarcinoma sections. **A**) H&E stain showing prostatic adenocarcinoma. **B**) Positive AMACR immunoreactivity, with arrows indicating areas of robust staining. **C**) Negative ERG immunoreactivity. **D**) AR immunoreactivity with arrows highlighting strong expression areas.

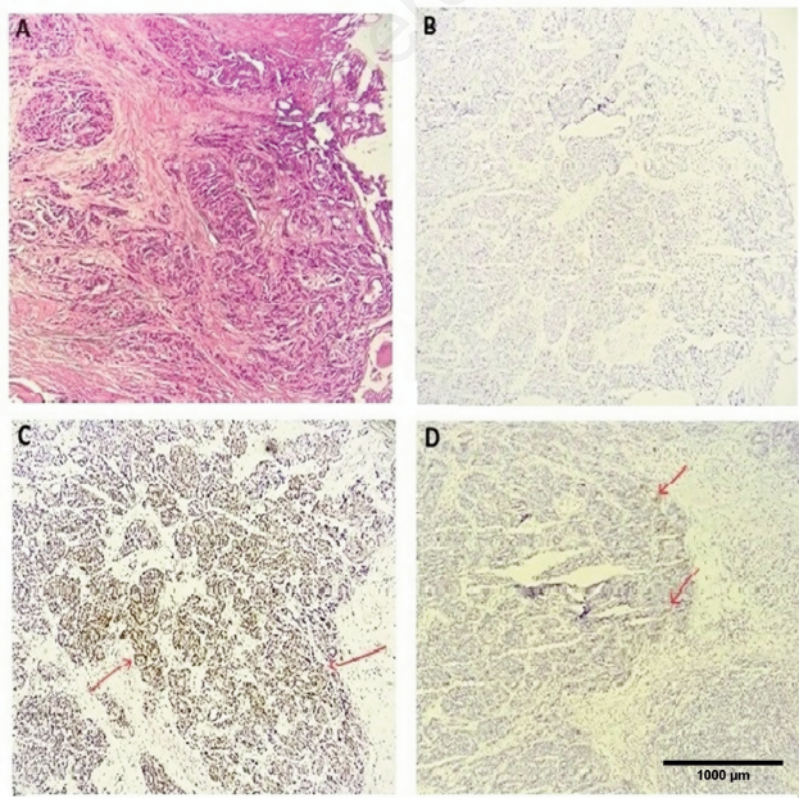

Figure 3. Histological examination of prostatic adenocarcinoma. **A**) H&E stain revealing the architectural features of prostatic adenocarcinoma. **B**) Negative AMACR immunoreactivity. **C**) Positive ERG immunoreactivity, with arrows indicating the areas of strong nuclear staining. **D**) AR immunoreactivity, with arrows highlighting regions of positive expression in the epithelial cells. .

ERG immunopositivity in 38-45% of Pca, 22-29% of HGPIN, and rare expression in benign glands.[29,30] The current study revealed an ERG immunostain sensitivity of only 35.29% but with a significantly high specificity of 80%. Lower sensitivity in the present study may be attributed to tumor heterogeneity and older patients, as ERG heterogeneity is 57% in elders' patients with PCa,[31] and the samples are almost of needle biopsies. The carcinoma belonging to GG1- GG3 did not take up the stain, but among GG4and GG5 cases, 6 out of 12 cases (50%) showed positivity. Amidst these 3 (50%) cases demonstrated a 3+ expression score with strong intensity. Our ERG positivity of 35.29% is in correlation with the study of Tsoulakis *et al*., who noted 32.2% positivity.[31] Mosquera *et al*.[32] noted ERG positivity of 27.8% in PCa and observed a higher positivity rate in lower GS with only 13.6% among GG5. Verdu *et al*.[33] noted no significant association between ERG expression and Gleason score. Mannan *et al*.[34] found a significant increase in ERG expression with higher GS but showed lower intensity with increasing GS. In addition, recent work by Feitosa *et al*.[35] suggests that patients with high ERG expression intensity are significantly more likely to develop biochemical relapse, metastasis, and cancer-specific mortality. Their study of 635 samples found that moderate to strong ERG staining correlated with higher cancer staging. Notably, while 41% of ERG-positive patients developed metastasis, no statistically significant difference in metastasis rates was observed based on ERG expression intensity. This highlights the potential prognostic significance of ERG immunoexpression in PCa and reinforces the value of including ERG as a marker within our diagnostic framework.

The variations of expression in different series might be related to racial differences as the studies were from different continents. The specificity is observed to be high as only 1 out of 5 cases of benign prostatic hyperplasia is immunoreactive with ERG, but it showed 1+ expression with weak intensity.

AR immunostain demonstrated a very high sensitivity of 94.12%, but with no specificity as all benign cases were also noted to be positive. AR expression was 3+ in 5 out of 6 cases among GG1-GG4, whereas 3+ was expressed in only 3 out of 10 cases of GG5. The intensity of stain was also higher in GG1-GG4 (4/6 cases showed strong), with only 1 case showing strong intensity in GG5. Mosquera *et al*.[32] reported 87.3% AR positivity in PCa, with 27.5% positivity in GG1 and 20.3% in GG5. In most cases, the stain's intensity was weak to moderate, with only a few 3+ cases among higher GS. They also noted co-expression of ERGand AR in 64.7% of lower GS PCa and 35.3% of higher GS cases. Their 75% benign lesions showed AR positivity.[36] We observed co-expression (ERG+/AR+) in all 6 cases of ERG-positive PCa. Of higher GS. Our benign cases revealed only mild to moderate expression and low intensity, except in one case, which showed 3+ with moderate intensity. The ERG correlation with higher GS in the present study is the opposite of Mosquera *et al*.,[32] though it correlates with other studies.[37,38] The AR staining was inversely proportional to PCa grade groups in this study, like that of the Indian study of Husain *et al*.[39] While comparing all three immunohistochemical stains in prostate carcinoma, it is observed that sensitivity and specificity vary significantly among these stains. AMACR appears more sensitive, and ERG appears more specific. Both these stains show a progressive increase in both expression as well as intensity with higher Gleason score carcinoma and higher-grade groups, especially in GG5. The interpretation of AMACR immunoreactivity needs to be correlated with respect to malignancy histologic features as the weak intensity with a low expression score tends to be seen in benign and low-grade malignancy. The ERG immunoreactivity is more indicative of carcinoma though it expressed weak positivity only in one benign lesion. There is a progressive increase in expression as well as the intensity of ERG with higher-grade groups of carcinomas. The previous studies revealed a varying incidence of TMPRSS2-ERG gene fusion among different races, about 50% in Caucasians, 31% in African Americans, and 16% in Asians.[32,40] The specificity of ERG may be further enhanced by using FISH technology for ERG gene rearrangement as weak immunostain with low expression in <10% cells are likely to be FISH negative. Tomlins *et al*. demonstrated 100% specificity of ERG gene arrangement in PCa by FISH-based analysis.[41] The utility of AMACR alone in diagnosing PCa may give rise to false-positive and false-negative results. AR immunostain expression score and intensity of staining are inversely proportional to AMACR and ERG in relation to the grade of carcinoma. Though AR itself is not specific to carcinoma, this stain may help to detect metastatic prostatic carcinoma. Our results suggest that AMACR and ERG immunostains may serve as valuable diagnostic and prognostic tools in prostatic adenocarcinoma. These findings align with previous studies and underscore the need for further investigation into additional biomarkers that may enhance diagnostic accuracy. Future research should focus on the clinical outcomes related to immunostain expression levels and explore potential therapeutic implications to improve patient management.

Although this study offers valuable insights, there are limitations to consider. First, our study is restricted to three immunostains (AMACR, ERG, and AR) and does not discuss other potentially actionable biomarkers that may improve diagnostic accuracy and prognostic assessment. There may be further studies including the broader range of biomarkers to present the more holistic view of PCa. Second, this study did not correlate the immunostaining expression levels with clinical outcomes, which would have enhanced the implication of the immunostains expression level on patient prognosis. Confirmations of such correlations in future studies would clarify the clinical relevance of these markers. Finally, the relatively small sample size of 25 biopsies limits the ability to extrapolate our findings to a broader population. While our results provide valuable insights into the utility of AMACR, ERG, and AR immunostains in diagnosing and prognosticating PCa, larger studies are required to confirm these findings and enhance their applicability across diverse patient populations. Future research should aim to include a more extensive cohort to strengthen the validity and reliability of the results.

In conclusion, the immunochemical stains of AMACR and ERG when used together are of utmost significance in evaluating PCa for diagnosis as the former is more sensitive and the latter is more specific. They are also valuable in prognosis as their expression is stronger in higher GG of PCa and the ERG positive molecular type appears more aggressive than the ERG negative. AR may also be of prognostic significance as its expression is lower in higher GG among PCa, though it is not much of diagnostic value in the prostate's primary adenocarcinoma. Nevertheless, AR immunostain may be utilized in the interpretation of metastatic adenocarcinoma in men. We recommend i) to take caution while interpreting 1+ weak intensity AMACR immunoreactivity and correlate with histologic findings and/or basal cell markers; ii) to detect ERG gene expression using FISH in selected cases with low ERG immunostain score and intensity.

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